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13. ABSTRACT (Maximum 200 Words)

TRAIL is a tumor necrosis factor family member that can specifically induce apoptosis of cancer cells but not of normal cells. However, some cancer cells are resistant to TRAIL-induced apoptosis. The purpose of this proposed study is to clone and characterize such inhibitory genes of TRAIL-induced apoptosis. Using cDNA subtraction and retroviral cDNA-based expression cloning approaches, we have obtained more than mutiple candidate clones of TRAIL-inhibitory genes. Among the candidate clones, the short splice form of Casper/c-FLIP (Casper-S_ were shown to confer resistance to TRAIL-induced apoptosis. Casper deficient embryonic cells were sensitive to TRAIL-induced apoptosis. Re-introduction of Casper-S into Casper deficient cells conferred resistance to TRAIL-induced apoptosis. This project has identified and validated Casper-S as a major cellular inhibitor of TRAIL-induced apoptosis.

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INTRODUCTION

TRAIL is a tumor necrosis factor family member that can specifically induce apoptosis of cancer cells but not of normal cells (1-5). However, some cancer cells are resistant to TRAIL-induced apoptosis (3, 4, 6-13). The purpose of this proposed study is to clone and characterize such inhibitory genes of TRAIL-induced apoptosis. To this end, following specific aims are proposed: #1. To clone genes that inhibit TRAIL-induced apoptosis of MCF7 cells by a subtractive hybridization screening approach and an expression/functional cloning approach using retroviral cDNA libraries; #2. To functionally characterize genes identified from specific aim #1.

BODY

We proposed the following Tasks and time frames in the original proposal (Table I):

Table I: Proposed Tasks

Task 1. To clone genes which inhibit TRAIL-induced apoptosis of MCF7 cells by a subtractive hybridization screening approach and an expression/functional cloning approach using retroviral cDNA libraries (months 1-24)

a. Isolate and amplify TRAIL resistant (MCF7-R) and sensitive (MCF7-S) MCF7 cells (months 1-2)
b. Identify genes that inhibit TRAIL-induced apoptosis of MCF7 cells by a subtractive hybridization screening approach (months 3-12)
c. Confirm that identified candidate genes are differentially expressed in MCF7-S and MCF-R cells Northern blot analysis (months 13-15)
d. Identify genes that inhibit TRAIL-induced apoptosis by an expression/functional cloning approach using retroviral cDNA libraries (months 3-20)
e. Clone full length cDNAs for genes that are identified from Task 1 (months 21-24)

Task 2. To functionally characterize genes identified from *Task 1* (months 25-36)

a. Determine tissue distribution of expression of the genes cloned in *Task 1* (months 25-26).
b. Test whether these genes can inhibit TRAIL-induced apoptosis in apoptosis assays (months 25-28)
c. Determine the molecular mechanisms responsible for the inhibition of TRAIL-induced apoptosis by the genes identified in *Task 1* (months 29-36)

Work Done in this project:

We isolated TRAIL-sensitive (TS) and resistant (TR) cells. We then purified mRNAs from TS and TR cells and performed PCR-based cDNA subtractive hybridization

experiments. Based on this approach, we have identified 9 genes from a forward subtraction (TR minus TS) and one gene from a reverse subtraction (TS minus TR). The identities of these genes are listed in TABLE II. (Task 1a, 1b).

TABLE II. Identities of bands from the cDNA subtraction

Forward Subtraction

Band #	Identfity/Reference
1	PP2Ac (Protein Phosphatase 2A Catalytic Subunit)
2	Casper/c-FLIP
3	CL100/DUSP1 (Dual Specific Phosphatase)
4	FIP-1/RagA (Ras-related GTPase)
5	Mitochodrial ATP Synthase
6	RAN (Ras family member)
7	Novel
8	Novel
9	Novel

Reverse Subtraction

1	TRAIL-R2

We also performed retroviral cDNA library based functional cloning of TRAIL resistant genes. We used two commercially available libraries, a human leukocyte and a fetal liver retroviral libraries, to infect TS cells by retroviral-mediated gene transfer techniques. After infection, we treated the infected cells with TRAIL and have obtained ~20 true TRAIL-resistant clones. The sequence identities of the cDNA inserts in the retroviral vectors in the resistant clones are shown in TABEL III. (Task 1d).

Table III. Identities of the 52 Sequenced Clones from Functional Screenings

Clone ID	Identification (Number of Clones Obtained)
C1	Casper/c-FLIP(17)
C2	PP2Ac (protein phosphatase 2A Catalytic Subunit)(2)
C3	NALP1/CARD7 (1)
C4	MKK4 (1)
C5	SerpinA3 (serine proteinase inhibitor, clade A, member 3)(1)
C6	IL2-R(interleukin 2 receptor)(1)
C7	NOS3 (nitric oxide synthase 3)(1)
C8	SPTLC1 (serine palmitoyltransferase)(1)
C9	Albumin (5)
C10	Novel (1)
C11	Novel (2)
C12	Novel (1)
C13	Novel (2)
C14	Novel (2)
C15	Beta-hemoglobin (3)
C16	Beta-actin (2)

Other clones only contains 3' UTR of various genes

Very interestingly, among the 52 sequenced clones, 17 encode for Casper/c-FLIP. Further sequencing analysis suggests that all 17 Casper clones represent the short splice form of Casper (Casper-S). Using transient transfection and stable transfection approaches, we firstly confirmed that Casper-S could confer resistance to TRAIL sensitive cells. Furthermore, we found that Casper deficient embryonic fibroblasts (EFs) were highly sensitive while their wild-type counterparts were completely resistant to TRAIL-induced apoptosis. Retroviral-mediated transduction of Casper-S into Casper(-/-) EFs restored resistance to TRAIL. These data suggest that Casper-S/c-FLIPs is a major cellular inhibitor of TRAIL-induced apoptosis. Our studies on Casper-S in TRAIL resistance were published (Bin et al., FEBS Letter, 2002, 510:37-40). (Task 1e, 2b, 2c).

Our results on Casper-S indicated that our approaches for identification of TRAIL resistant genes are working properly, and we validated the first TRAIL resistant genes identified in this project.

We further analyzed the other candidate genes obtained from the expression cloning. We made retroviral expression plasmids for 12 candidate genes. To do this, we amplified cDNAs for these genes by PCR using a mixed cDNA libraries or the isolated clones as templates. The cDNAs were inserted into the pFB-Neo retroviral plasmid (Stratagene). The identities and related information are summarized in Table IV.

Table IV

Candidate genes	Known functions	References
PP2Ac	Protein phosphatase	15
CL100/DUSP1	Dual specific phosphatase	16-17
FIP-1/RagA	Ras-related GTPase	18
RAN	Ras family member	19
NALP1/CARD7	CARD domain-containing protein	20
MKK4	MAP kinase kinase	21
α1-antiproteinase	Serine proteinase inhibitor	22
NOS3	Nitric oxidase synthase	23
SPTLC1	Serine palmitoyltransferase	24
C10	Novel	
C11	Novel	
C13	Novel	

We transduced these genes into TRAIL sensitive cell clone HC1 by retroviral-mediated gene transfer. Two days after infection with the retrovirus containing these genes, cells were treated with TRAIL for overnight. We used 200 ng/ml of TRAIL for the treatment, a concentration we used in the previous expression cloning. We found that retroviral mediated transfer of the tested genes did not confer resistance to TRAIL-induced apoptosis, as judged by observation under a microscope. (Task 1e, 2b, 2c).

Because the novel genes identified in this project could not confer resistance to TRAIL-induced apoptosis, we did not detect the tissue distribution of mRNA expression of these genes (Task 2a).

KEY RESEARCH ACCOMPLISHMENTS

- Isolated and amplified TRAIL resistant (TR) and sensitive (TS) cells.
- Identified 9 candidate TRAIL-inhibitory clones by subtractive hybridization screening experiments.
- Identified 52 candidate clones of TRAIL-inhibitory genes by expression/functional cloning experiments using retroviral cDNA libraries.
- Sequenced and analyzed all candidate clones obtained from expression/functional cloning experiments using retroviral cDNA libraries.
- Identified and characterized Casper-S as a major inhibitor of TRAIL-induced apoptosis.
- Cloned 12 candidate genes into retroviral vector and determined whether they could inhibit TRAIL-induced apoptosis.

REPORTABLE OUTCOMES

One paper was published:

Bin, L., Li, X., Xu, L., Shu, H.B. 2002. The short splice form of Casper/c-FLIP is a major cellular inhibitor of TRAIL-induced apoptosis. FEBS Letter 510:37-40.

CONCLUSIONS

We have performed most of the proposed experiments. We identified the short splice form of Casper is the major cellular inhibitor of TRAIL-induced apoptosis.

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APPENDICES

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The short splice form of Casper/c-FLIP is a major cellular inhibitor of TRAIL-induced apoptosis

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Abstract TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) is a member of the tumor necrosis factor family that selectively induces apoptosis of cancer cells. However, some cancer cells or subpopulations within cancer cell lines are resistant to TRAIL-induced apoptosis. We developed a retroviral cDNA library-based functional cloning approach to unambiguously identify putative inhibitory genes of TRAIL-induced apoptosis. This effort identified the short splice form of Casper/c-FLIP, Casper-S/c-FLIPs, as a major cellular protein that confers resistance to TRAIL-induced apoptosis. Furthermore, we found that Casper deficient embryonic fibroblasts (EFs) were highly sensitive while their wild-type counterparts were completely resistant to TRAIL-induced apoptosis. Retroviral-mediated transduction of Casper-S/c-FLIPs into Casper (−/−) EFs restored resistance to TRAIL. These data suggest that Casper-S/c-FLIPs is a major cellular inhibitor of TRAIL-induced apoptosis.

Key words: TNF-related apoptosis-inducing ligand; Casper-S/c-FLIPs; Apoptosis; Functional cloning; Inhibitor

1. Introduction

TRAIL (tumor necrosis factor (TNF)-related apoptosis-inducing ligand) is a recently identified member of the TNF family that selectively induces apoptosis of cancer cells [1,2]. In mouse and primate models, TRAIL has been shown to cause reduction in tumor growth rate and in some cases complete elimination of tumors without detectable toxicity [3,4], pointing to the possibility of developing TRAIL as a reagent for cancer treatment.

TRAIL induces apoptosis through two death domain-containing receptors, TRAIL-R1/DR4 and TRAIL-R2/DR5 [5,6]. Two additional receptors, TRAIL-R3/TRID/DcR1 and TRAIL-R4 also bind to TRAIL [5,6]. TRAIL-R3 is a GPI-linked receptor that does not have a cytoplasmic domain, while TRAIL-R4 has a cytoplasmic domain containing one-third of the consensus death domain motif. It has been suggested that TRAIL-R3 and TRAIL-R4 function as 'decoy' receptors to inhibit TRAIL-induced apoptosis [7,8].

Although TRAIL can selectively induce apoptosis of certain cancer cells, some cancer cells or subpopulations within cancer cell lines are resistant to TRAIL. The molecular mechanisms responsible for cells' resistance to TRAIL are controversial. Although it has been suggested that a cell's resistance to TRAIL-induced apoptosis might be caused by expression of the 'decoy' receptors TRAIL-R3 and TRAIL-R4 [7,8], several careful studies indicate that this is not the case, or at least is not the only mechanism. In these studies, numerous cancer cell lines were analyzed for their sensitivity to TRAIL in the context of expression of different TRAIL receptors. These studies indicated that there was no correlation between the expression of potential 'decoy' receptors TRAIL-R3 and/or TRAIL-R4 and a cell's sensitivity to TRAIL [5,6,9,10].

Another potential mechanism responsible for a cell's resistance to TRAIL involves the activation of necrosis factor (NF)-κB. Previously, several studies have established that NF-κB activation can protect cells from TNF-induced apoptosis, probably through its ability to induce the expression of anti-apoptosis genes [11]. Recently, several studies indicated that activation of NF-κB conferred resistance to TRAIL-induced apoptosis [12,13]. However, these studies can not exclude the possibility that NF-κB-independent proteins can also inhibit TRAIL-induced apoptosis. In fact, other studies showed that activation of NF-κB was not sufficient to block TRAIL-induced apoptosis in certain cell types [14,15]. Thus, both NF-κB-dependent and -independent proteins may be involved in cells' resistance to TRAIL-induced apoptosis.

To get a better understanding on the mechanisms of TRAIL resistance, which is important to develop TRAIL into a cancer drug, we have developed a retroviral cDNA library-based functional cloning approach to unambiguously identify genes that inhibit TRAIL-induced apoptosis. Our findings indicate that the short splice form of Casper/c-FLIP (Casper-S/c-FLIPs) is a major cellular inhibitor of TRAIL-induced apoptosis.

2. Materials and methods

2.1. Reagents and cell lines

Recombinant human soluble TRAIL (aa 95–291) was produced in *Escherichia coli* and purified by our own laboratory. The retroviral human leukocyte cDNA library (Clontech) and fetal liver cDNA library (Stratagene), and the packaging cell line 293-10A1 (Imgenex) were purchased from the indicated manufacturers. HeLa cells were provided by Dr. David Riches (National Jewish Medical and Research Center). The mouse Casper(−/−) and wild-type embryonic

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fibroblasts (EFs) were previously described [16]. All cells described above were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

2.2. Constructs

Casper/c-FLIP and Casper-S/c-FLIPs retroviral plasmids were constructed by insertion of their respective cDNAs into the retroviral vector pFB-Neo (Stratagene).

2.2.1. MTT assays. Cells ($\sim 2\times10^5$) were cultured in 6-well dishes and treated with various concentration of TRAIL for 6 h. The apoptotic cells were then washed off by PBS and the survival cells were incubated in fresh medium with 0.5 mg/ml of MTT (Sigma) for 6 h. The brown MTT-derivative crystals were dissolved in 1 ml of 70% isopropanol/0.02 N HCl, and the values at OD_{570} were read by a spectrophotometer.

2.3. Isolation of TRAIL-sensitive HeLa clones

HeLa cells were seeded in 100 mm dishes at a density of ~ 50 cells/dish. Two weeks later, individual clones were pick up into 24-well dishes. One week after seeding, cells from each well were split into two wells. Cells in one well were treated with 100 ng/ml of TRAIL for 6 h. Only clones that all cells died were defined as TRAIL-sensitive clones and were used for further experiments.

2.4. Cell transfection and retroviral-mediated gene transfer

Cells were transfected by the standard calcium phosphate precipitation method. Retroviral-mediated gene transfer was performed following the recommended procedures by the manufacturers (Imgenex and Clontech).

To screen for genes that inhibit TRAIL-induced apoptosis, 293-10A1 cells ($\sim 2\times10^6$/dish) were transfected with 15 μg of human leukocyte or fetal liver retroviral cDNA library plasmids by calcium phosphate precipitation. 18 h later, the cells were washed with PBS and cultured in 5 ml of fresh medium. 24 h later, the recombinant retrovirus-containing medium was collected and centrifuged. The supernatant, supplemented with 4 μg/ml polybrene, was used to infect TRAIL-sensitive HC1 (a HeLa subclone) cells ($\sim 2\times10^6$). Two days after infection, the cells were treated with 200 ng/ml of TRAIL for 24 h, and then subjected to two more rounds of TRAIL treatment. TRAIL-resistant clones were pick up and amplified. Genomic DNAs of the resistant clones were extracted and the inserts of the retroviral vectors integrated into the resistant clones were amplified by PCR and sequenced. In these screening experiments, a total of $\sim 2\times10^7$ cells were infected and 52 TRAIL-resistant clones were obtained and sequenced.

2.5. Western blot

Western blot was performed with a rabbit polyclonal antibody recognizing both the long and short forms of Casper [17].

3. Results and discussion

To identify genes that inhibit TRAIL-induced apoptosis, we have used HeLa cells as a model system. As shown in Fig. 1A, TRAIL could induce apoptosis of HeLa cells in a dose-dependent manner. At a concentration of 200 ng/ml, TRAIL could kill approximately 95% of treated HeLa cells in 6 h. However, $\sim 5\%$ of treated HeLa cells were resistant (Fig. 1A), and these cells remained resistant even when TRAIL concentration was increased to 1000 ng/ml and the treatment time was extended to 48 h (data not shown). To make sure the resistant phenotype is stable, we amplified the resistant cells and performed five more rounds of treatment with a high concentration of TRAIL (1000 ng/ml). After these treatments, the cells were completely resistant to TRAIL (Fig. 1B). We designated these TRAIL-resistant cells as HeLa-TR.

There are at least two possible mechanisms that may account for HeLa-TR cells' resistance to TRAIL. Firstly, one or more intracellular signaling components of TRAIL-induced apoptosis pathway are absent in the resistant cells; alternatively, one or more inhibitory proteins are expressed in the resistant cells but not or at low levels in the sensitive cells, and these inhibitory proteins can inhibit TRAIL-induced apoptosis. To distinguish these two possibilities, we examined the effect of cycloheximide, a protein synthesis inhibitor, on HeLa-TR cells' response to TRAIL. We found that in the presence of 2 μg/ml of cycloheximide, TRAIL (200 ng/ml) could induce apoptosis of almost 100% of treated HeLa-TR cells in 6 h (Fig. 1B). In the same experiments, TRAIL or cycloheximide alone had no apoptosis-inducing effect on HeLa-TR cells (Fig. 1B). Similar results were observed with several other TRAIL-resistant cancer cell lines (data not shown). These data suggest that the signaling components of TRAIL-induced apoptosis pathway are intact in the resistant cells and that one or more short-lived inhibitory proteins expressed in the resistant cells are responsible for resistance to TRAIL-induced apoptosis.

To identify inhibitory genes of TRAIL-induced apoptosis in resistant cells, we developed a retroviral cDNA library-based functional cloning approach. The procedures are illustrated in

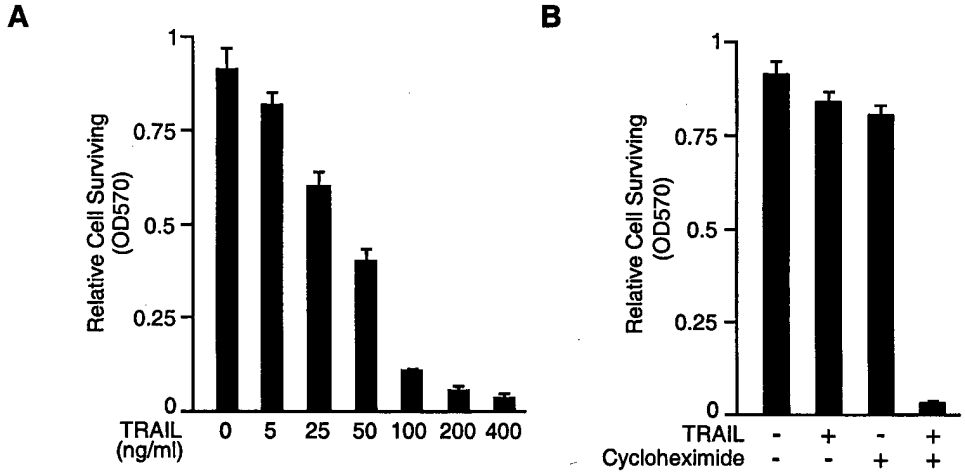

Fig. 1. A: TRAIL induces apoptosis of HeLa cells in a dose-dependent manner. HeLa cells were treated with the indicated amounts of TRAIL for 6 h and the relative cell survival was measured by MTT assays. B: Conversion of TRAIL-resistant cells to sensitive cells by cycloheximide. HeLa-TR cells were treated with TRAIL (200 ng/ml), cycloheximide (2 μg/ml), or both together for 6 h and the relative cell survival was then measured by MTT assays.

Fig. 2. Briefly, we firstly isolated a HeLa subclone (HC1) that is highly sensitive to TRAIL-induced apoptosis. At a concentration of 200 ng/ml, TRAIL could kill ~100% of treated HC1 cells in 4 h (data not shown). To screen for TRAIL-inhibitory genes, we transfected ~5×10^7 of 293-10A1 packaging cells with ~150 μg of retroviral human leukocyte and fetal liver cDNA library plasmids by calcium phosphate precipitation. Since normal cells are resistant to TRAIL [5,6], they should be ideal sources for potential TRAIL-inhibitory genes. Therefore, we performed our functional cloning experiments with the two commercially available retroviral cDNA libraries.

Two days after transfection of the 293-10A1 packaging cells with the retroviral cDNA library plasmids, recombinant retrovirus-containing medium was collected and used to infect ~2×10^7 HC1 cells. The infected HC1 cells were subjected to three rounds of treatment with TRAIL (200 ng/ml). The TRAIL-resistant clones were amplified and the integrated inserts in the retroviral vector were amplified by PCR. Sequencing analysis indicates that 16 out of 52 TRAIL-resistant clones encode Casper-S/c-FLIPs, which is corresponding to aa 1–202 of the full-length Casper/c-FLIP [17–20]. In addition to Casper-S/c-FLIPs, one of the TRAIL-resistant clones encodes a previously unidentified splice form of Casper/c-FLIP, which is corresponding to aa 1–267 of the full-length Casper/c-FLIP.

There are several possible reasons that the obtained clones are resistant to TRAIL. Firstly, the protein encoded by the cDNA insert in the retroviral vector can inhibit TRAIL-induced apoptosis; secondly, the retroviral vector may insert into and disrupt a gene required for TRAIL-induced apoptosis; thirdly, insertion of the retroviral vector may activate an endogenous TRAIL-inhibitory gene. In the latter two cases, the TRAIL-resistant phenotype of a clone is not caused by expressed protein encoded by the cDNA insert of the integrated retroviral vector.

To confirm that Casper-S/c-FLIPs can inhibit TRAIL-induced apoptosis, we made a retroviral vector for Casper-S/c-FLIPs. This vector was introduced into the TRAIL-sensitive HeLa clone by retroviral-mediated gene transfer and the transduced cells were selected by G418. The stable cell line was treated with TRAIL or left untreated, and MTT assay was performed to examine cell viability. These experiments indicated that retroviral-mediated gene transfer of Casper-S/c-FLIPs was sufficient to confer resistance to TRAIL-sensitive cells (Fig. 3). As expected, crmA, a specific caspase inhibitor, could also inhibit TRAIL-induced apoptosis (Fig. 3). Similar experiments suggest that the other candidate genes from the functional screening can not inhibit TRAIL-induced apoptosis and therefore represent artificial clones from the screenings.

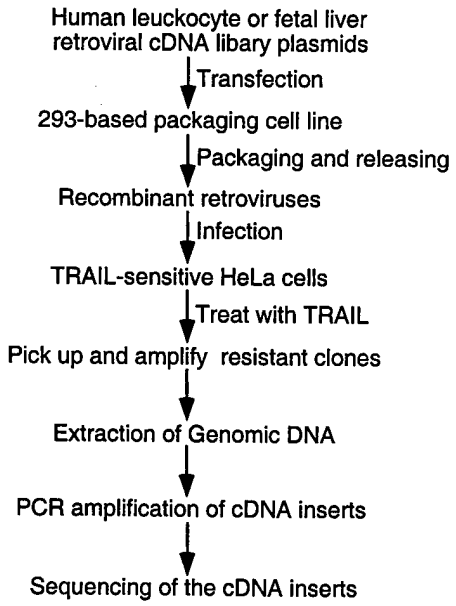

Fig. 2. Strategy for identification of TRAIL-inhibitory genes. See text for details.

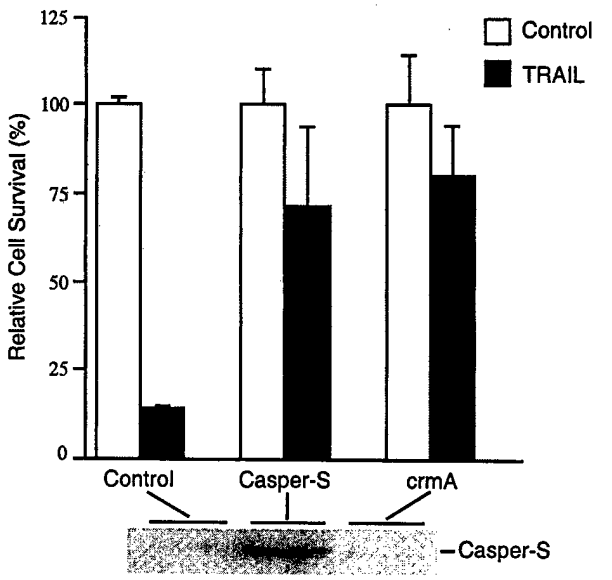

Fig. 3. Inhibition of TRAIL-induced apoptosis by ectopic expression of Casper-S/c-FLIPs. The TRAIL-sensitive HeLa cells were stably transduced with control, Casper-S/c-FLIPs, or crmA retroviral vectors. The stably transduced cells were treated with TRAIL (200 ng/ml) (black bars) or left untreated (white bars) for 6 h. Relative cell survival was then measured by MTT assays. The protein levels of Casper-S/c-FLIPs in the transduced cells were examined by Western blot (lower panel).

Casper/c-FLIP was identified as a FADD- and caspase-8-related protein [17–20]. Casper/c-FLIP contains two death effector domains and a caspase-like domain. Casper/c-FLIP is not a caspase because the conserved cysteine residue among all caspases is not present in Casper/c-FLIP. There are several alternative spliced transcripts detected in mammalian cells, but only two forms of Casper/c-FLIP can be detected at the protein level. These include the short form, Casper-S/c-FLIPs, which contains aa 1–202, and the full-length long form, which contains 480 aa. A viral protein, v-FLIP, is structurally related to the short form of Casper/c-FLIP and is a potent inhibitor of apoptosis [19,20].

Previously, it has been shown that overexpression of Casper-S/c-FLIPs can inhibit death receptor-mediated apoptosis. Overexpression of the full-length Casper/c-FLIP protein, however, can either induce apoptosis or inhibit apoptosis, probably depending on protein expression levels and cell types [17–20].

To further investigate Casper's role in TRAIL-induced ap-

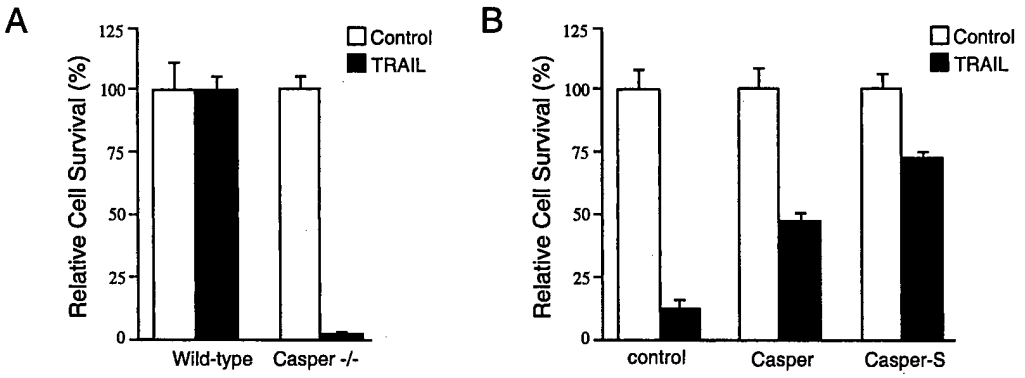

Fig. 4. Casper-S/c-FLIPs is critically involved in cells' resistance to TRAIL-induced apoptosis. A: Casper (−/−) but not wild-type EFs are sensitive to TRAIL-induced apoptosis. Casper(−/−) and wild-type EFs were treated with TRAIL (200 ng/ml) or left untreated for 6 h. Relative cell survival was then measured by MTT assays. B: Re-introduction of Casper-S/c-FLIPs into Casper(−/−) EFs restores resistance to TRAIL. Casper-S/c-FLIPs and the full-length Casper/c-FLIP were re-introduced into Casper(−/−) EFs by retroviral-mediated gene transfer, then treated with TRAIL (200 ng/ml) or left untreated for 6 h. Relative cell viability was then measured by MTT assays.

optosis, we examined the effect of TRAIL on Casper/c-FLIP gene knock-out EFs, referred hereafter as Casper(−/−) EFs. We found that Casper(−/−) EFs were highly sensitive, while their wild-type counterparts were completely resistant to TRAIL-induced apoptosis (Fig. 4A). These data suggest that Casper/c-FLIP is required and sufficient for EF cells' resistance to TRAIL-induced apoptosis. Since Casper(−/−) EFs do not express either Casper-S/c-FLIPs or the full-length Casper/c-FLIP [16], we determined their individual contribution to EF cells' resistance to TRAIL-induced apoptosis. To do this, we re-introduced them into Casper(−/−) EFs by retroviral-mediated gene transfer and found that both of them could significantly restore resistance to TRAIL-induced apoptosis (Fig. 4B). However, Casper-S/c-FLIPs was more potent than the full-length Casper/c-FLIP in inhibiting TRAIL-induced apoptosis in Casper(−/−) EFs in these experiments. This is consistent with the fact that we only isolated Casper-S/c-FLIPs but not the full-length Casper/c-FLIP in our functional cloning experiments. This is also consistent with some recent studies suggesting that Casper-S/c-FLIPs is a more potent inhibitor of apoptosis than the full-length Casper/c-FLIP in various systems [21,22].

In conclusion, our findings suggest that Casper-S/c-FLIPs is a major cyto-protective protein of TRAIL-induced apoptosis.

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